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PRINCIPAL INVESTIGATOR: Xiankai Sun

CONTRACTING ORGANIZATION: University of Texas
Dallas, TX 75390

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Xiankai Sun

E-Mail: xiankai.sun@utsouthwestern.edu

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14. ABSTRACT

Polyarginines are a group of small peptides that have been used as drug delivery vehicles due to their capability of penetrating cell membranes. In one of our studies using such a peptide to deliver a therapeutic moiety to various prostate cancer cell lines, we surprisingly discovered that the peptide had remarkably high preference to prostate tissues. This specificity, which has not been reported before, prompted us to exploit this group of peptides for the early detection of prostate tumor metastases. Promisingly, in our preliminary studies, the peptide labeled with 64Cu can clearly reveal metastases in a tumor-bearing animal model. In this one year period, we demonstrate that the uptake of NH2GR11 is mediated through macropinocytosis. However, inhibitors for carbohydrate synthesis of glycoprotein via either O-link or N-link, or inhibitor for carbohydrate-chain elongation of glycosaminoglycan did not alter NH2GR11 uptake by prostate cells. In contrast, laminin receptor, a PentS binding partner, was able to influence NH2GR11 uptake in prostate cancer cells. Thus, we conclude that laminin receptor is one of initial binding site(s) responsible for R NH2GR11 peptide uptake in prostate cells.

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Introduction

The ultimate goal of this project is to develop specific PET imaging probes for early detection of distal metastases of prostate cancer. Based on one of our studies using a polyarginine (NH_2GR11) to deliver a therapeutic moiety to various prostate cancer cell lines, we hypothesize a new group of prostate-specific peptides could be developed as novel PET imaging probes using NH_2GR11 as lead compound for the detection of multi-foci extraprostatic spread of prostate cancer. Three specific aims are arranged to achieve the goals of this proposal: (I) Determine the mechanism of the prostate-specific uptake exhibited by NH_2GR11; (II) Design and synthesize novel BFC-peptide conjugates in order to achieve desired in vivo stability, pharmacokinetics, and enhanced prostate-specific binding affinity; and (III) Establish radiochemical protocols to label peptide conjugates with ^{64}Cu, and perform in vitro/*in vivo* evaluations of the potential prostate cancer-specific imaging agents.

Body

As shown in my 1st and 2nd annual reports and the reviewer's comments, our research had progressed well in the first two years. However in the 3rd year, we have met major technical problems in the mouse model establishment, which impeded our work to accomplish Task 8 in Aim III.

Task 8 (Months 24 – 36): PET Imaging Evaluation of Radiolabeled Peptides in PCa Tumor-Bearing Mice.

Early detection of metastatic PCa tumor growth. We anticipate 2 peptides with desired properties for this task. Four tumor-bearing animal models will be established using osteolytic PC-3-Luc and osteoblastic C4-2-Luc cell lines (subcutaneous and intra-femur). To evaluate each selected peptide in each animal model, we will use 6 mice bearing tumors (at three different stages: 2, 4, and 6 weeks after tumor-cell injection; n = 2) by comparing to FDG as control standard. Thus 72 nude mice are needed for this study (6 × 3 (2 peptides plus FDG) × 4). Prior to the PET imaging, the animals will be imaged by BLI to assess the tumor growth. The imaging time points will be judiciously chosen according to the biodistribution studies. The images will be analyzed quantitatively to evaluate the potential application of the imaging agents for the early detection of extraprostatic PCa. After the imaging of the final time point, the animals will be sacrificed to perform post-PET biodistribution studies, whose results will be used as golden standards for the imaging evaluation.

PCa detection specificity as compared to other carcinomas. We will use the subcutaneous tumor model to evaluate the PCa specificity of the imaging probes by comparing with seven other carcinoma cell lines. The left flank of animals will be implanted with C4-2 or PC-3 tumor cells, while the right flank with other cancer cell lines (Lung cancer: H1299 and A549; kidney cancer: SW839 and A-498; bladder cancer: T24 and 253J; and Liver cancer: HepG2). A group of 2 animals will be used for the comparison to each of the cell lines. Therefore, it requires 28 nude mice (2 × 7 × 2). From 1 week post tumor implantation, PET imaging studies will be performed weekly with the imaging probes (i.v.) in one month. The images will be analyzed quantitatively to evaluate the imaging probe specificity in the detection of PCa *vs* other tumors. We anticipate the extraprostatic PCa spread could be differentiated from other cancer metastases by our imaging probes.

The technical problems were:

1. The C4-2-Luc cell line does not consistently induce osteoblastic bone metastases. We have tracked back to the original stock of the cell line. It appears that multiple passaging of the cells might be the problem. We are now re-establishing the osteoblastic model.

2. The growth rates of different tumors are hard to control. We expected to use a mouse model bearing two tumor xenografts with a similar size for the imaging specificity evaluation; however the dual-tumor model was not successfully established for all the proposed tumor cell lines. While still continuously pursuing the dual-tumor model, we plan to use the single tumor model for each different tumor type to complete the proposed experiments in the worst scenario.

Therefore, we requested a 12-month no-cost extension to focus on our efforts on the establishment of the two tumor models (1 – 6 months); and then complete the proposed imaging experiments to accomplish the Task 8 (7 – 12 months) as specified in Aim III of the project.

In addition to the trials of animal tumor model establishment, in the 3rd year we have spent significant effort on the further elucidation of the NH_2GR11 prostate targeting mechanism.

Experimental Methods and Materials

Materials and Methods

Cell line and Reagents: The PNT1A cell line, an immortalized prostate epithelial cell line, was cultured in RPMI1640 supplemented with 10% Fetal Bovine Serum (FBS). Three prostate cancer cell lines (LNCaP, C4-2 and PC3) were cultured as described previously (Zhou et al. 2005). CHO-K1 and PgsA-745 cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained in Kaighn's modification of Ham's F12 medium (F12K) supplemented with 10% FBS. All the oligo-arginine peptides were made by automated peptide synthesizer using the standard solid phase chemistry from our core facility and purified by reverse phase HPLC. In this study, the purity of these peptides was more than 97% determined by HPLC and MALDI-TOF mass spectrometry (see Supplementary information). Benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside (BG), Swainsonine (SW) and Clostridium difficile Toxin B (Toxin B) was purchased from CalBiochem (Gibbstown, NJ). Heparan sulfate (HS), Dextran sulfate (DS), Chondroitin sulfate A (CS-A), Chondroitin sulfate B (CS-B), Chondroitin 6-sulfate (Chondroitin sulfate C, CS-C), 4-Nitrophenyl β-D-xylopyranoside (Xy) and EIPA was from Sigma-Aldrich (St. Louis, MO). Pentosan sulfate was synthesized from Xylan (poly(β-D-xylopyranose[1->4]); Sigma-Aldrich) by a patented method[footnote1]. After purification by size-exclusion HPLC, the pooled fractions were pooled and lyophilized to yield a puffy white powder. The molecular weight of pentosan sulfate was in the range of 3,000 – 10,000 Dalton, and its sulfur content was 14 – 15% as measured by barium sulfate precipitation analysis. Oligo-arginine peptides were synthesized in the core facility of our institute and were fluorescent tagged at the N-terminus as described previously (Zhou et al. 2006). 37LRP siRNA was purchased from Qiagen (Valencia, CA).

Measurement of uptake of oligo-arginine peptides: Cells (10^4 cell per well) were plated in 96-well plate for 48 h. FITC-tagged peptides with or without inhibitors were added into each well with 0.1 ml of RPMI 1640 containing 1% FBS. After 30 min incubation, the uptake was terminated by washing once with Phosphate Buffered Saline (PBS) containing Trypan Blue (0.4%) for 4 min and then three times with PBS. The fluorescent intensity of cell lysate (in 25 mM Tris pH 7.4, 150mM NaCl, 1% Triton X100) was detected using M5 plate reader (molecular Devices, Sunnyvale, CA) with Excitation 490 nm, Emission 530 nm, Cut-off 515 nm. The protein concentration of each sample was determined in the same plate using BCA protein assay kit (Pierce, Rockford, IL). The uptake was calculated after normalizing with the protein concentration of each well.

Determination of cellular distribution of CPP: To determine the subcellular localization of CPP, cells were washed three times with PBS and once with Trypan Blue (0.4% in PBS) then fixed with 4% paraformaldehyde in PBS and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) and examined under fluorescence microscope.

Transfection of siRNA and western blot analysis: Cells were plated in 96-well plate for 24 h then transfected with siRNA using Lipofectamine RNAimax (Invitrogen, Carlsbad, CA). Four days after transfection, the effect of siRNA was determined based on both the uptake of CPP and Western Blot analysis with Laminin receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis: All numerical data were expressed as mean ± standard deviation (SD). Statistical significance was determined by conducting a paired Student's *t* test. Results with p value less than 0.05 were considered statistically significant.

Results

The uptake efficiency of oligo-arginine peptides with different lengths: Previously, we have shown that the oligo-arginine peptide R11 has the highest uptake efficiency in prostate cells out of several CPPs (Zhou et al. 2006). It is also known that changing the length of the peptides affects uptake efficiency as well (Tyagi et al. 2001). Thus, different lengths of oligo-arginine peptides were synthesized (i.e., R9, R11 and R13) and their uptake were examined in several prostate cell lines derived from either normal (i.e., PNT1A) or cancer (i.e., LNCaP, C4-2 and PC3). The uptake efficiency was determined based on the fluorescence intensity of FITC conjugated with each peptide. The unbound peptides were washed with PBS and the external fluorescent signal was quenched by Trypan Blue for 4 min to reduce background from outside of the cells. As shown in Figure 1A, R11 peptide exhibited the highest uptake efficiency in all four cell lines tested. Of the other oligo-arginine peptides tested, R9 peptide had the second highest uptake efficiency (about 57-80% of R11 uptake) while R13 had the lowest uptake (4-15% of R11). This suggests that longer or shorter length oligo-arginine peptides could reduce their uptake efficiency in prostate cells. Fluorescence microscopy was used to determine the subcellular localization of R9, R11 and R13 in prostate cells and data indicated that the majority of peptides were localized in the cytosol exhibiting punctuated pattern (Figure 1B). Consistent with the uptake efficiency results, R11 peptide exhibited the highest fluorescent intensity among the three peptides tested in the four prostate cell lines

The route(s) of R11 uptake: To delineate the route of R11 peptide uptake, several inhibitors for different pathways were employed in this study. EIPA is an inhibitor for macrpinocytosis (Nakase et al. 2007). BG and SW are inhibitors to block the carbohydrate chain attachment to glycoprotein via either O-link or N-link respectively (Gala and Morrison 2002). Xy is known to block the carbohydrate chain elongation of glycosaminoglycan (Keller et al. 2008). Toxin B inhibits low-molecular-mass GTPase Rho, which is involved in endocytosis (Nakase et al. 2007). As shown in Figure 2A, treatment with EIPA significantly inhibited R11 uptake in PC3 cells, suggesting the involvement of macropinocytosis for the uptake of R11 in prostate cells. EIPA inhibited R11 uptake in prostate cells in a dose-dependent manner (Figure 2B). On the other hand, inhibitors of carbohydrate synthesis for either glycoprotein (BG and SW) or glycosaminoglycan (Xy), had minimal to no effect on R11 uptake (Figure 2A). Similar results were observed in several repeats of the experiment with different time courses. (data not shown). This suggests that R11 does not bind to the carbohydrate moiety on glycoprotein during cell entry. Also, Toxin B, an inhibitor of low-molecular-mass GTPase Rho, has shown little to no effect on R11 uptake, indicating that the involvement of Rho protein in the uptake of R11 peptide by prostate cells may be minimal.
GAGs and anionic polymers have been shown to affect CPP uptake (Tyagi et al. 2001; Richard et al. 2005; Nakase et al. 2007). To examine the involvement of GAGs, soluble GAGs such as CS-A, CS-B and CS-C were employed. In addition to GAGs, anionic polymers such as Heparan sulfate (HS) and pentosan sulfate

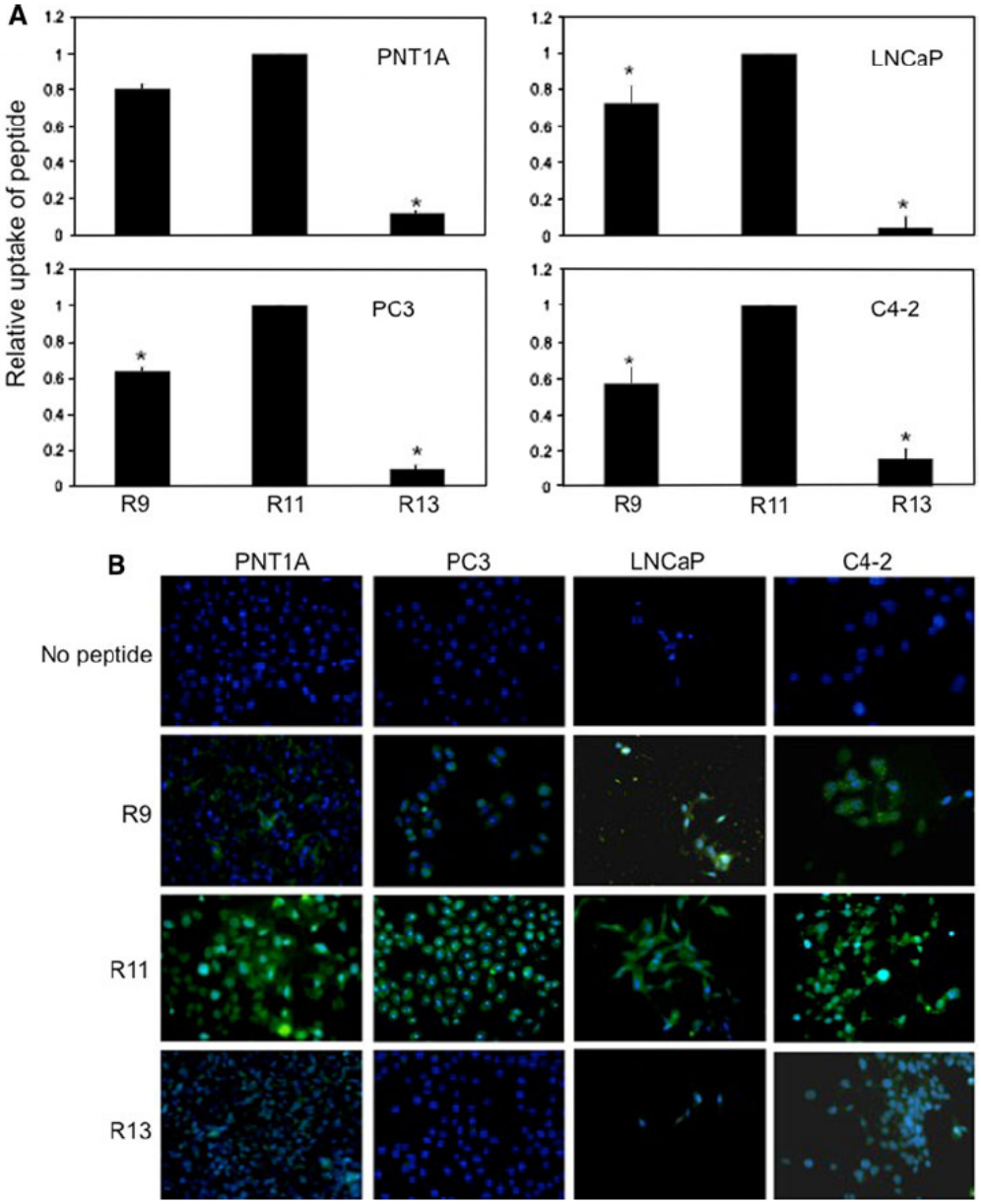

Fig. 1 Uptake of oligo-arginine peptides in prostate cells. **a** R9, R11 and R13 (5 µmol/L) were incubated with different cells for 30 min prior to harvesting. Relative FITC intensity from each sample was determined by normalizing fluorescence intensity with its protein concentration. Relative uptake of peptide in each cell was calculated using R11 = 1. *Columns* mean in triplicate; *bars* SD. All the experiments were repeated at least twice. **b** Cells were incubated with 5 µmol/L of each peptide for 30 min. After fixation, cells were counterstained with DAPI. The cellular distribution of each peptide was visualized with fluorescence microscope. *Significant difference between CPP and R11 ($P < 0.05$)

(PentS) were tested. Using a competitive assay, the soluble GAGs and anionic polymers were co-incubated with R11 peptide. As shown in Figure 3A, all compounds tested could inhibit the uptake of R11 peptide in PC3 cells in a dose-dependent manner. The degree of inhibition was: PentS > CS-B > HS ≈ CS-C > CS-A. Since PentS was the most potent, they were further tested in other prostate cells. Similar patterns of inhibition were observed for PentS (Figure 3B). This inhibitory effect can also be observed by using pre-incubating these compounds (Figure 3C). The data demonstrates that the interaction of GAG with cell surface molecules is critical for R11 peptide uptake by prostate cells, which suggests the presence of similar binding sites between GAG and R11 on cell surface molecules.

Data from Figure 2A indicated that the inhibition of carbohydrate synthesis for either glycoprotein or GAG using various inhibitors did not have significant effects on R11 peptide uptake by prostate cells. Thus, we believe that the inhibitory effect of soluble GAGs is most likely due to the competition between soluble GAGs and R11 peptide on the cell surface.

The role of 37LRP in R11 uptake: To further identify the "receptor" or binding protein for R11, we have searched possible candidate proteins for PentS for the most potent competitor. It appears that PentS is able to bind to laminin receptor (Vana et al. 2009); Laminin receptor in cell has a precursor form (37LRP) and a

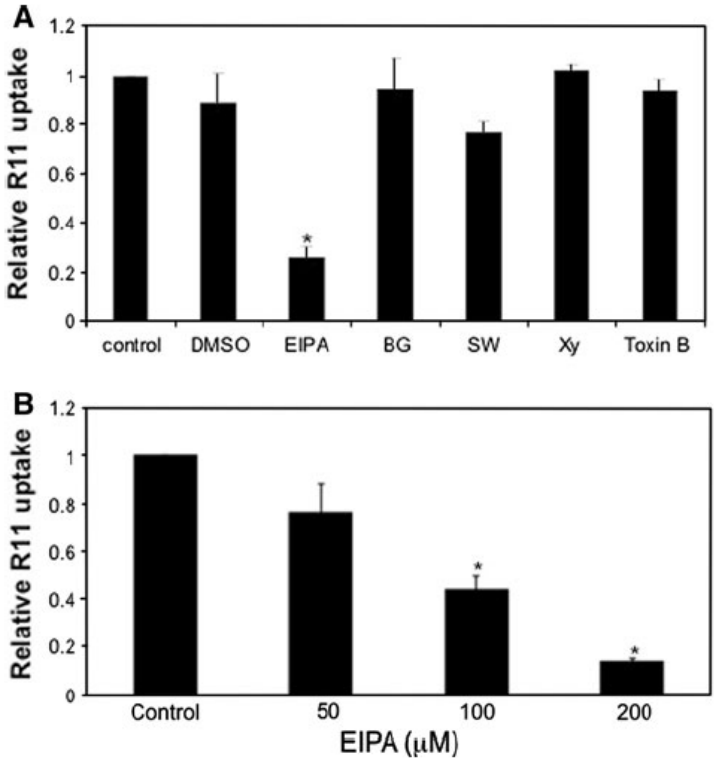

Fig. 2 Characterization of mechanism of R11 uptake. **a** Uptake of R11 was determined in PC3 cells pretreated with inhibitors then incubated with 5 μmol/L of R11. The specific inhibitors include EIPA (200 mM; 30 min), benzyl-2-acetamido-2-deoxy-α-BG (10 mg/mL; 48 h), SW (5 mg/mL; 48 h), Xy (5 mM; 48 h) and Toxin B (100 ng/mL; 24 h). **b** The effect of EIPA on R11 uptake in PC3 cells by pre-treating cells with EIPA for 30 min then adding 5 μmol/L of R11. Relative R11 uptake from each sample was determined by FITC intensity divided by its protein concentration and then normalized with control. *Columns* mean in triplicate; *bars* SD. *Significant difference between control and each treatment ($P < 0.05$)

mature form (67LP) (Nelson et al. 2008). The role of 37LRP in R11 peptide uptake was tested. In several prostate cell lines examined, we only observed 37LRP but not 67LR as the major band from western analyses, which may be due to two possibilities that 67LR is dissociated into 37LRP in the reducing condition during gel electrophoresis or 67LR expresses very low amount in prostate cancer cells. Thus, we examined whether R11 is able to associate with 37LRP in prostate cells. As shown in Figure 4A, after incubating cells with FITC-R11 and then immunoprecipitaing 37LRP using a specific antibody, it appeared that the binding of FITC-R11 to 37LRP exhibited a dose-dependent manner and this interaction was diminished by increasing amount of competitive R11. In addition, the binding affinity of each oligo-arginine to 37LRP (Figure 4B) was parallel with its cellular uptake (Figure 1A). These data suggest that 37LRP is one of primary "receptor" for R11 uptake by prostate cells. Furthermore, 37LRP level in prostate cells was further manipulated using gene specific siRNA. All four prostate cells were transiently transfected with 37LRP-specific siRNA and its control siRNA; mock transfection was used as a control. The efficiency of gene knockdown in each cell was determined using western blot analysis of 37LRP expression levels after normalizing with a house keeping protein (i.e., HSC70) then compared with that of mock transfection. As shown in Figure 5, a wide range of reduced 37LRP protein levels (20-80%) were present among these cell lines. R11 uptake was also determined from the same sample and was found to be 35-60% that of the mock transfection. The data suggests that 37LRP is one of the receptor(s) or a binding protein responsible for the uptake of R11 peptide by prostate cells.

R11 appears to be a highly efficient cell delivery vehicle compared with other CPPs in prostate cancers (Zhou et al. 2006) and an imaging probe for detecting metastatic prostate cells (Hao et al. 2010). It is also consistent with other findings that arginine-rich peptides exhibit higher delivery efficiency than other cationic homopolymers, such as histidine, ornithine and lysine (Mitchell et al. 2000). This higher efficiency has been attributed to the presence of the guanidine head group. In this study, we have found that R11 peptide has the highest uptake efficiency compared to R9 and R13 peptide in all prostate cells tested, including normal and cancer cells. We further analyzed the mechanism of R11 uptake in prostate cells. While the mechanism(s) of uptake of CPPs is not fully understood; two possible mechanisms have been proposed. The non-endocytosis mechanism is proposed based on the observation that the uptake of CPP is in energy- or temperature-independent manner (Futaki et al. 2007). Also, using liposome model system, CPP has been shown to cross the lipid bilayer, which supports non-endocytosis mechanism (Thoren et al. 2000). On the other hand, endocytosis mechanism is based on the observations that the uptake of CPP exhibits a punctuated pattern inside of the cell resembling cell internalization, and the

uptake of CPP is sensitive to endocytosis inhibitors (Futaki et al. 2007). However, recent studies have indicated that both mechanisms are not mutually exclusive (Fretz et al. 2007; Futaki et al. 2007). The contribution of either mechanism may depend upon the several factors such as cell types, individual CPP, culture condition and the physical/chemical property of payload. The subcellular distribution of R11 peptide with cytosolic or punctuated pattern (Figure 1B) leads us to suspect that both mechanisms may also co-exist in prostate cells. Nevertheless, based on the inhibitory effect of EIPA on R11 uptake in prostate cells (Figure 2), endocytosis mechanism should play a major role in this event.

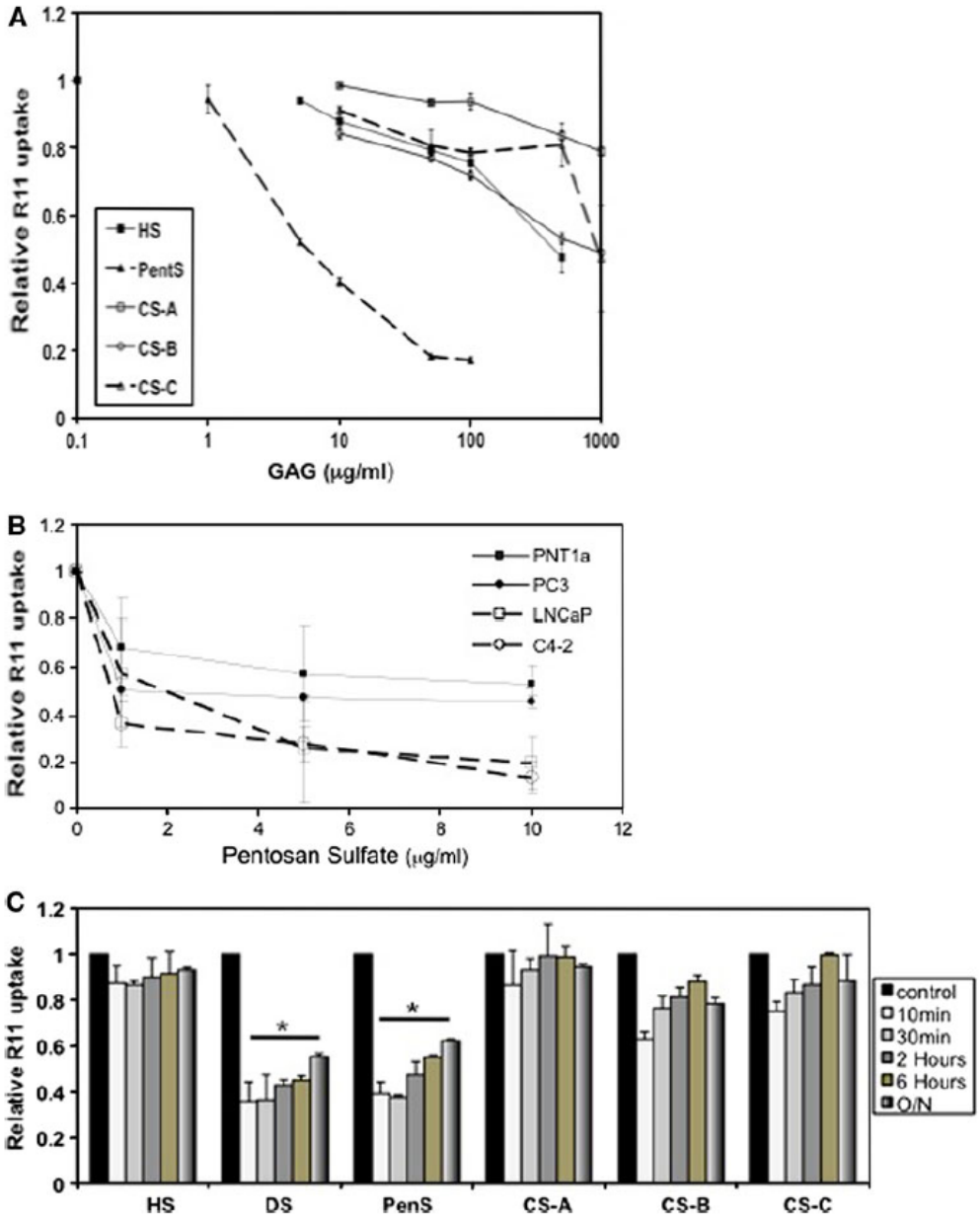

Fig. 3 The effect of soluble GAGs on R11 uptake in prostate cell lines. **a** Relative uptakes of R11 in PC3 cells was determined by co-incubating soluble glycosaminoglycans (GAGs) with 5 μmol/L of R11 for 30 min. **b** Effect of pentosan sulfate (PentS) on R11 uptake in various prostate cell lines. **c** Effect of GAGs on R11 uptake in various prostate cell lines by pre-incubation at different times. Different concentrations of GAGs were co-incubated with 5 μmol/L of R11 for 30 min. Relative R11 uptake from each sample was determined by FITC intensity divided by its protein concentration and then normalized with control. *Columns* mean in triplicate; *bars* SD. *Significant difference between control and each treatment ($P < 0.05$)

Apparently, GAGs can affect the uptake of CPP. In the presence of soluble GAGs, the uptake of TAT protein is significantly inhibited (Tyagi et al. 2001; Nakase et al. 2007), suggesting that this inhibition is due to the competition of CPP binding between soluble and cell surface GAGs. Also, the membrane-associated GAGs, such as heparin sulfate proteoglycan (HPSG) are important for uptake of Tat peptide (Tyagi et al. 2001; Richard et al. 2005; Nakase et al. 2007). Pre-incubation of anionic polymers, such as dextran sulfate, can increase the uptake of oligo-lysine peptide (Tyagi et al. 2001). However, there is another indication that some CPPs, such as R8 peptide, are less dependent on HSPG, implying the

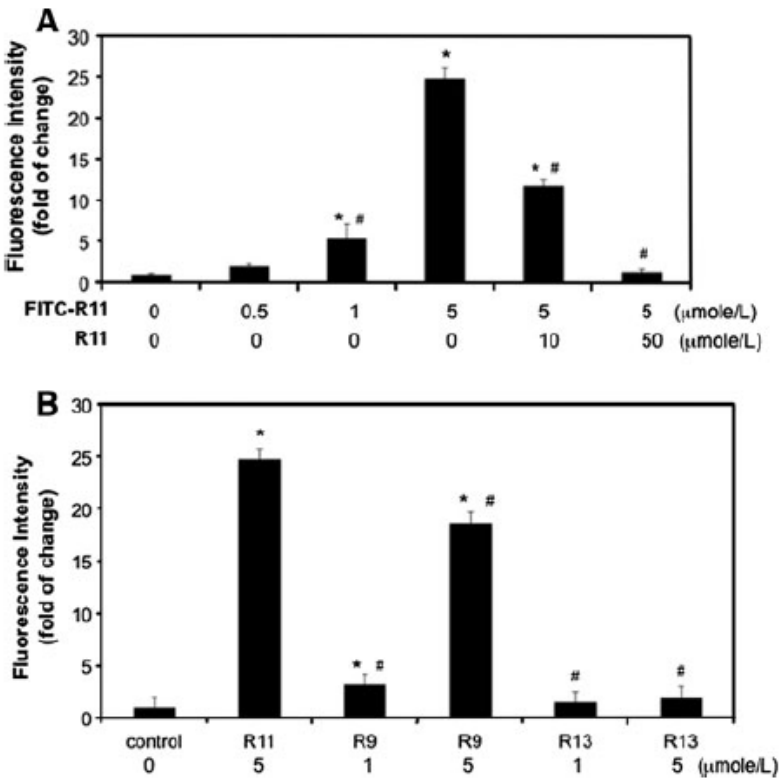

Fig. 4 The binding of R11 to 37LRP. **a** FITC-R11 or fluorescein was incubated with C4-2 cell line for 30 min incubation in the presence of competitive R11. **b** Various FITC-CPPs or fluorescein was incubated with C4-2 cell line for 30 min of incubation. After incubation, an equal amount of cell lysate (30 μg) was prepared from each treatment and subjected to immunoprecipitation with 37LRP antibody, and the immunocomplex was then determined by fluorometry. Relative fluorescence intensity from each sample was calculated by subtracting from control IgG and then normalizing with fluorescein control (=1). *Significant difference between control and each treatment ($P < 0.05$). #Significant difference between R11 (5 μmol/L) and each treatment ($P < 0.05$)

existence of alternative cell surface binding site (Nakase et al. 2007). Our study has clearly demonstrated that the carbohydrate moiety of glycoproteins or carbohydrate-chain elongation of proteoglycan failed to block R11 peptide uptake by prostate cells (Figures 2), which further supports the idea that the cell surface binding site(s) for R11 peptide is very similar to GAGs interactive site(s).

The 37-kDa laminin receptor precursor (i.e., 37LRP) has been shown to bind to laminin and it can facilitate the interaction between laminin and integrins (Nelson et al. 2008). In addition, 37LRP protein has been suggested to play a role in the internalization of microorganism, as well as peptide and proteins. Some bacteria and viruses use the laminin receptor to enter into mammalian cells (Wang et al. 1992; Kim et al. 2005; Tio et al. 2005). The laminin receptor has been found to interact with prion protein identified from a yeast two-hybrid screening and that his interaction can be blocked by HS or PentS (Rieger et al. 1997). Moreover, laminin receptor has been shown to be a cell surface binding site for the internalization of the prion protein (Gauczynski et al. 2001). Interestingly, 37LRP is also involved in the binding of a CPP, PCK3145, that is derived from prostate secretory protein 94 (PSP94), as determined by a chemical analysis for the binding protein of the PCK3145 peptide (Panchal et al. 2006). Our data indicates that R11 peptide uptake can be inhibited by soluble PentS (Figure 3) that is known to inhibit binding of 37LRP towards prion protein. Also, R11 can bind to 37LRP (Figure 4) and R11 peptide uptake can be inhibited by 37LRP-specific siRNA in all the prostate cells tested (Figure 5). Apparently, 37LRP is a primary binding molecule for R11 peptide uptake by prostate cells. Nevertheless, we cannot rule out the possibility of additional binding proteins responsible for this uptake because the observed inhibition of R11 uptake was not proportional to the reduced 37LRP expression levels in PC3 or LNCaP cell line (Figure 5B and C).

Certainly, the uptake of CPP is mediated through complicated mechanisms, which are highly dependent on the cell type, the composition of CPP, and perhaps its payload. We showed that the uptake of R11 peptide by prostate cells can be mediated via 37LRP by macropinocytosis. The entry of CPP peptide in vivo will be affected by the local extracellular micro-environments such as the composition of GAGs as well as the administration of the pharmaceutical reagents including carbohydrates such as PentS and HS. The results obtained from this study provide valuable information to determine whether R11 can be a potential delivery system for any given target cell or tissue. Also, understanding of uptake mechanism of R11 should help the development of its application for any specific biomolecules.

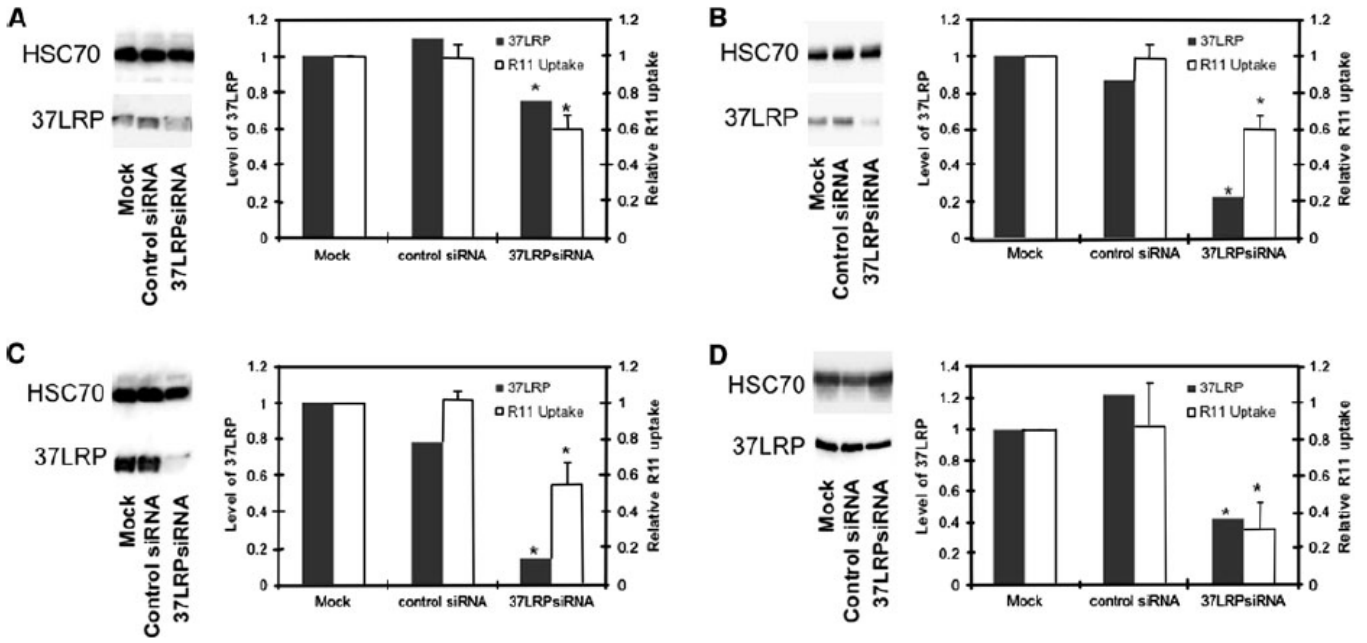

Fig. 5 The role of 37LRP in R11 uptake. PNT1A (**a**), PC3 (**b**), LNCaP (**c**) and C4-2 (**d**) cells were transfected with 37LRP siRNA or control siRNA for 4 days. R11 peptide (5 μmol/L) was added to each cell line for 30 min of incubation. The expression of 37LRP was determined using Western blot analysis and HSC70 was used as the internal control. The 37LRP protein level was quantified after normalizing with an internal control. The relative 37LRP protein level was calculated from each sample in triplicate after normalizing with the mock transfection. Relative R11 uptake from each sample in triplicate was determined by FITC intensity divided by its protein concentration and then normalized with mock transfection. *Bars* SD. *Significant difference between mock and each siRNA ($P < 0.05$)

Key Research Accomplishments

The laminin receptor is one of initial binding site(s) responsible for R11 peptide uptake in prostate cells.

Reportable Outcomes

1. Zhou J, Liu W, Pong RC , Hao G, Sun X, and Hsieh JT: Analysis of oligo-arginine cell-permeable peptides uptake by prostate cells. *Amino Acids*. In press. DOI:10.1007/s00726-010-0817-7.
2. Hao G, Singh A, Gore C, Hsieh JT, and Sun X: Presenting a cell permeable peptide on a chelator scaffold for PET imaging of prostate cancer. *The Annual Meeting of the Society of Nuclear Medicine*, 2011, June 3rd – 8th , San Antonio, TX (Accepted for oral presentation)

Conclusions

Cell permeable peptide is considered as an efficient delivery system to cross the cell membrane barrier. However, the underlying mechanisms are very complicated. Oligo-arginine peptide (i.e., R11) appears to have high affinity to prostate cancer cells compared with other peptides tested. We are able to develop R11 as an imaging probe to detect prostate cancer. In this study, we demonstrate that the uptake of R11 is mediated through macropinocytosis. However, inhibitors for carbohydrate synthesis of glycoprotein via either O-link or N-link, or inhibitor for carbohydrate-chain elongation of glycosaminoglycan did not alter R11 uptake by prostate cells. In contrast, laminin receptor, a PentS binding partner, was able to influence R11 uptake in prostate cancer cells. Thus, we conclude that laminin receptor is one of initial binding site(s) responsible for R11 peptide uptake in prostate cells.

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ORIGINAL ARTICLE

Analysis of oligo-arginine cell-permeable peptides uptake by prostate cells

Jian Zhou · Wei Liu · Rey-Chen Pong · Guiyang Hao · Xiankai Sun · Jer-Tsong Hsieh

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Abstract Recently, we have shown that oligo-arginine peptide (i.e., R11), a unique cell-permeable peptide (CPP), can be used as an imaging probe for prostate cancer detection. In this study, the mechanism(s) of oligo-arginine peptide in prostate cells was further analyzed. The length of the oligo-arginine peptide appears to be critical for the efficiency of uptake by prostate cells: poly (11)-arginine (R11) > poly (9)-arginine (R9) > poly (13)-arginine peptide (R13). The uptake of R11 peptide by prostate cells is mediated by macropinocytosis as evidenced by the fact that uptake can be blocked by a macropinocytosis inhibitor. However, the use of an inhibitor for carbohydrate chain elongation of glycosaminoglycan or inhibitors for carbohydrate synthesis of glycoprotein via either O-link or N-link showed minimal effects on R11 uptake. Nevertheless, pentosan sulfate (PentS) or dextran sulfate (DS) exhibited the highest inhibitory effect on R11 uptake in several prostate cells treated with various soluble glycosaminoglycans (GAGs) or anionic polymers. It is known that laminin receptor has been characterized as a PentS binding partner. Knocking down 37LRP (laminin receptor precursor) expression in prostate cells showed a reduction in their ability to uptake R11 peptides. In conclusion, laminin receptor is one of the initial binding site(s) responsible for R11 peptide uptake in prostate cells.

Keywords Oligo-arginine peptide · Cell-permeable peptide · Laminin receptor · Prostate cells

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J. Zhou · R.-C. Pong · J.-T. Hsieh (✉)
Department of Urology, J8-134, UT Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA
e-mail: jt.hsieh@utsouthwestern.edu

W. Liu · G. Hao · X. Sun
Department of Radiology, UT Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA

X. Sun
Department of Advance Imaging Research Center, UT Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA

J.-T. Hsieh
Graduate Institute of Cancer Biology, China Medical University and Hospital, Taichung 404, Taiwan, Republic of China

Introduction

The plasma membrane defines the boundary of the cell and functions as an impermeable barrier for most macromolecules. However, some proteins such as HIV-1 Tat protein, *Drosophila melanogaster* Antennapedia protein and fibroblast growth factor 1 and 2 (Frankel and Pabo 1988; Joliot et al. 1991; Malecki et al. 2004) are capable of crossing plasma membrane and entering into the cell. It appears that the protein domain related to this function, the protein transduction domain (PTD), contains a short stretch of basic amino acids. PTD-containing peptides that are capable of crossing the plasma membrane are referred to as cell-permeable peptides (CPPs). Currently, there are two types of CPPs: one is derived from native proteins such as penetratin peptide from Antennapedia protein and Tat peptide from HIV-1 Tat protein; the other is synthetic such as oligo-arginine, oligo-lysine and KALA peptides. CPPs become promising delivery vehicles for bioactive molecules, such as small interfering RNA, DNA, peptides or protein in vitro and in vivo (Wender et al. 2000).

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The exact mechanism of CPP uptake by the cell is still very controversial (Futaki et al. 2007). Direct penetration of the membrane by CPPs has been proposed and is based on studies from biological and biophysical experiments. The most striking evidence is that CPPs can translocate liposomes, a model lipid bilayer (Thoren et al. 2000). On the other hand, some studies have shown that endocytosis plays a major role in the uptake of penetratin, oligo-arginine, TAT peptide and HIV-1 Tat protein (Drin et al. 2003; Richard et al. 2003; Vendeville et al. 2004). Inhibitors for endocytosis, particularly 5-ethylisopropyl amiloride (EIPA), an inhibitor of macropinocytosis, have been shown to reduce the uptake of CPP. Nevertheless, the initial and critical step is considered to be the association of CPP with the plasma membrane, since the uptake of CPP by cells can be inhibited by soluble GAGs in the culture medium (Tyagi et al. 2001; Richard et al. 2005).

We have recently shown that the oligo-arginine peptide, R11, has the highest uptake efficiency in prostate cells compared with other CPPs (TAT, KALA, penetratin and oligo-lysine) (Zhou et al. 2006). Using R11 as a delivery vehicle, the introduction of the proline-rich domain derived from DOC-2/DAB2, a tumor suppressor protein, could inhibit the proliferation of prostate cancer cells by suppressing the MAP kinase pathway. In this study, we have studied possible uptake mechanisms and the efficiency of different oligo-arginine peptides in prostate cells. We found that the R11 peptide appeared to be more efficient than R9 or R13 and that its uptake is mediated through the interaction of laminin receptor protein via macropinocytosis in prostate cells.

Materials and methods

Cell line and reagents

The PNT1A cell line, an immortalized prostate epithelial cell line, was cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS). Three prostate cancer cell lines (LNCaP, C4-2 and PC3) were cultured as described previously (Zhou et al. 2005). CHO-K1 and PgsA-745 cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained in Kaighn's modification of Ham's F12 medium (F12K) supplemented with 10% FBS.

All the oligo-arginine peptides were made by automated peptide synthesizer using the standard solid phase chemistry from our core facility and purified by reverse phase HPLC. In this study, the purity of these peptides was more than 97% determined by HPLC and MALDI-TOF mass spectrometry (see Supplementary information).

Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BG), swainsonine (SW) and *Clostridium difficile* Toxin B (Toxin B) was purchased from CalBiochem (Gibbstown, NJ). Heparan sulfate (HS), dextran sulfate (DS), chondroitin sulfate A (CS-A), chondroitin sulfate B (CS-B), chondroitin 6-sulfate (chondroitin sulfate C, CS-C), 4-nitrophenyl β-D-xylopyranoside (Xy) and EIPA were from Sigma-Aldrich (St. Louis, MO). Pentosan sulfate was synthesized from xylan (poly(β-D-xylopyranose[1→4]); Sigma-Aldrich) by a patented method (Deshpande et al. 2008) After purification by size-exclusion HPLC, the pooled fractions were pooled and lyophilized to yield a puffy white powder. The molecular weight of pentosan sulfate was in the range of 3,000–10,000 Dalton, and its sulfur content was 14–15% as measured by barium sulfate precipitation analysis. Oligo-arginine peptides were synthesized in the core facility of our institute and were fluorescent tagged at the N-terminus as described previously (Zhou et al. 2006). 37LRP siRNA was purchased from Qiagen (Valencia, CA).

Measurement of uptake of oligo-arginine peptides

Cells (10^4 cells per well) were plated in a 96-well plate for 48 h. FITC-tagged peptides with or without inhibitors were added into each well with 0.1 mL of RPMI 1640 containing 1% FBS. After 30 min of incubation, the uptake was terminated by washing once with phosphate-buffered saline (PBS) containing Trypan Blue (0.4%) for 4 min and then three times with PBS. The fluorescent intensity of cell lysate (in 25 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X100) was detected using M5 plate reader (Molecular Devices, Sunnyvale, CA) with excitation 490 nm, emission 530 nm and cutoff 515 nm. The protein concentration of each sample was determined in the same plate using BCA protein assay kit (Pierce, Rockford, IL). The uptake was calculated after normalizing with the protein concentration of each well.

Determination of cellular distribution of CPP

To determine the subcellular localization of CPP, cells were washed three times with PBS and once with Trypan Blue (0.4% in PBS) then fixed with 4% paraformaldehyde in PBS and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) and examined under fluorescence microscope.

Transfection of siRNA and Western blot analysis

Cells were plated in a 96-well plate for 24 h then transfected with siRNA using Lipofectamine RNAimax (Invitrogen, Carlsbad, CA). Four days after transfection, the effect of siRNA was determined based on both the uptake of CPP and Western blot analysis with laminin receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis

All numerical data were expressed as mean ± standard deviation (SD). Statistical significance was determined by conducting a paired Student's *t* test. Results with *P* value <0.05 were considered statistically significant.

Results

The uptake efficiency of oligo-arginine peptides with different lengths

Previously, we have shown that the oligo-arginine peptide R11 has the highest uptake efficiency in prostate cells out of several CPPs (Zhou et al. 2006). It is also known that changing the length of the peptides affects uptake efficiency as well (Tyagi et al. 2001). Thus, different lengths of oligo-arginine peptides were synthesized (i.e., R9, R11 and R13) and their uptake were examined in several prostate cell lines derived from either normal (i.e., PNT1A) or cancer cells (i.e., LNCaP, C4-2 and PC3). The uptake efficiency was determined based on the fluorescence intensity of FITC conjugated with each peptide. The unbound peptides were washed with PBS and the external fluorescent signal was quenched by Trypan Blue for 4 min to reduce background from outside the cells. As shown in Fig. 1a, R11 peptide exhibited the highest uptake efficiency in all four cell lines tested. Of the other oligo-arginine peptides tested, R9 peptide had the second highest uptake efficiency (about 57–80% of R11 uptake), while R13 had the lowest uptake (4–15% of R11). This suggests

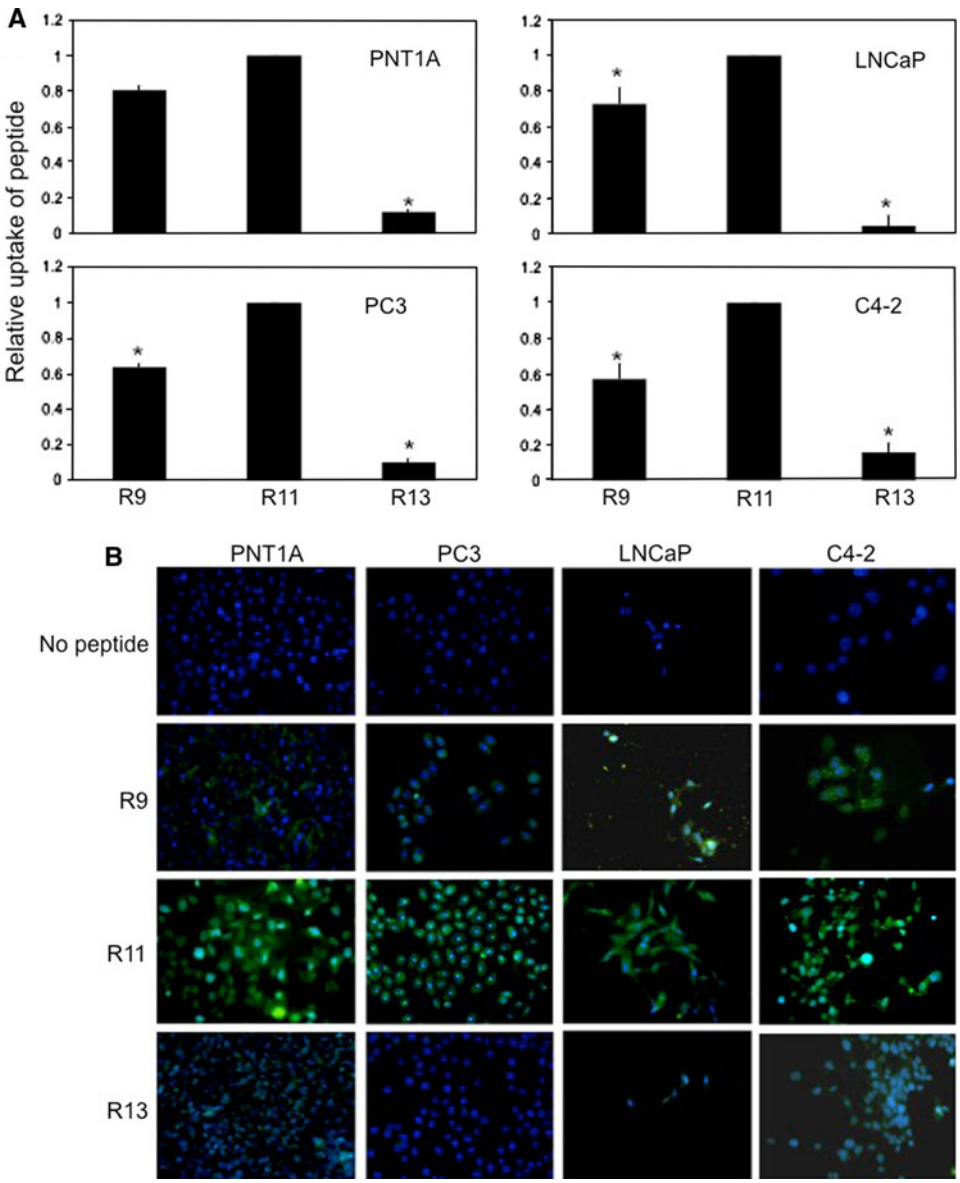

Fig. 1 Uptake of oligo-arginine peptides in prostate cells. **a** R9, R11 and R13 (5 μmol/L) were incubated with different cells for 30 min prior to harvesting. Relative FITC intensity from each sample was determined by normalizing fluorescence intensity with its protein concentration. Relative uptake of peptide in each cell was calculated using R11 = 1. *Columns* mean in triplicate; *bars* SD. All the experiments were repeated at least twice. **b** Cells were incubated with 5 μmol/L of each peptide for 30 min. After fixation, cells were counterstained with DAPI. The cellular distribution of each peptide was visualized with fluorescence microscope. *Significant difference between CPP and R11 ($P < 0.05$)

that longer or shorter length oligo-arginine peptides could reduce their uptake efficiency in prostate cells. Fluorescence microscopy was used to determine the subcellular localization of R9, R11 and R13 in prostate cells, and data indicated that the majority of peptides were localized in the cytosol exhibiting punctuated pattern (Fig. 1b). Consistent with the uptake efficiency results, R11 peptide exhibited the highest fluorescent intensity among the three peptides tested in the four prostate cell lines.

The route(s) of R11 uptake

To delineate the route of R11 peptide uptake, several inhibitors for different pathways were employed in this study. EIPA is an inhibitor for macrpinocytosis (Nakase et al. 2007). BG and SW are inhibitors that block the carbohydrate chain attachment to glycoprotein via either O-link or N-link, respectively (Gala and Morrison 2002). Xy is known to block the carbohydrate chain elongation of glycosaminoglycan (Keller et al. 2008). Toxin B inhibits low-molecular mass GTPase Rho, which is involved in endocytosis (Nakase et al. 2007). As shown in Fig. 2a,

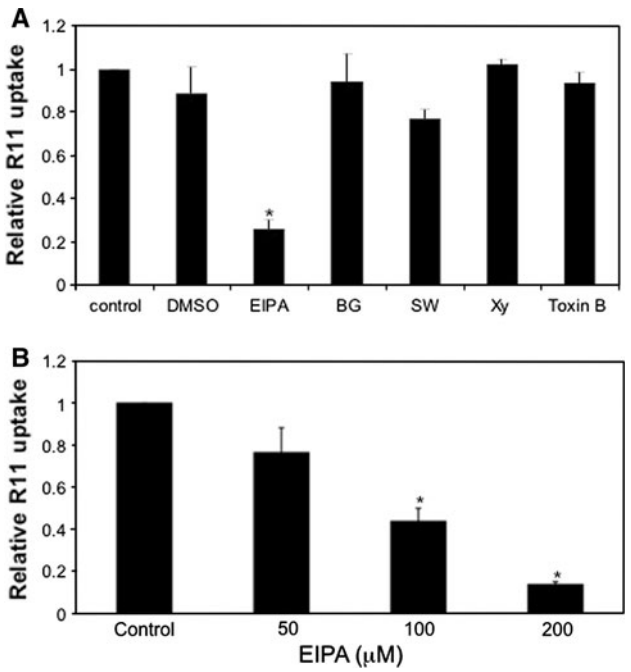

Fig. 2 Characterization of mechanism of R11 uptake. **a** Uptake of R11 was determined in PC3 cells pretreated with inhibitors then incubated with 5 μmol/L of R11. The specific inhibitors include EIPA (200 mM; 30 min), benzyl-2-acetamido-2-deoxy-α-BG (10 mg/mL; 48 h), SW (5 mg/mL; 48 h), Xy (5 mM; 48 h) and Toxin B (100 ng/mL; 24 h). **b** The effect of EIPA on R11 uptake in PC3 cells by pretreating cells with EIPA for 30 min then adding 5 μmol/L of R11. Relative R11 uptake from each sample was determined by FITC intensity divided by its protein concentration and then normalized with control. *Columns* mean in triplicate; *bars* SD. *Significant difference between control and each treatment ($P < 0.05$)

treatment with EIPA significantly inhibited R11 uptake in PC3 cells, suggesting the involvement of macropinocytosis for the uptake of R11 in prostate cells. EIPA inhibited R11 uptake in prostate cells in a dose-dependent manner (Fig. 2b). On the other hand, inhibitors of carbohydrate synthesis for either glycoprotein (BG and SW) or glycosaminoglycan (Xy) had minimal to no effect on R11 uptake (Fig. 2a). Similar results were observed in several repeats of the experiment with different time courses (data not shown). This suggests that R11 does not bind to the carbohydrate moiety on glycoprotein during cell entry. Also, toxin B, an inhibitor of low-molecular mass GTPase Rho, has shown little to no effect on R11 uptake, indicating that the involvement of Rho protein in the uptake of R11 peptide by prostate cells may be minimal.

GAGs and anionic polymers have been shown to affect CPP uptake (Tyagi et al. 2001; Richard et al. 2005; Nakase et al. 2007). To examine the involvement of GAGs, soluble GAGs such as CS-A, CS-B and CS-C were employed. In addition to GAGs, anionic polymers such as heparan sulfate (HS) and pentosan sulfate (PentS) were tested. Using a competitive assay, the soluble GAGs and anionic polymers were co-incubated with R11 peptide. As shown in Fig. 3a, all compounds tested could inhibit the uptake of R11 peptide in PC3 cells in a dose-dependent manner. The degree of inhibition was: PentS > CS-B > HS ≈ CS-C > CS-A. Since PentS was the most potent, they were further tested in other prostate cells. Similar patterns of inhibition were observed for PentS (Fig. 3b). This inhibitory effect can also be observed by using pre-incubating these compounds (Fig. 3c). The data demonstrate that the interaction of GAG with cell surface molecules is critical for R11 peptide uptake by prostate cells, which suggests the presence of similar binding sites between GAG and R11 on cell surface molecules.

Data from Fig. 2a indicated that the inhibition of carbohydrate synthesis for either glycoprotein or GAG using various inhibitors did not have significant effects on R11 peptide uptake by prostate cells. Thus, we believe that the inhibitory effect of soluble GAGs is most likely due to the competition between soluble GAGs and R11 peptide on the cell surface.

The role of 37LRP in R11 uptake

To further identify the "receptor" or binding protein for R11, we have searched possible candidate proteins for PentS for the most potent competitor. It appears that PentS is able to bind to laminin receptor (Vana et al. 2009). Laminin receptor in cell has a precursor form (37LRP) and a mature form (67LP) (Nelson et al. 2008). The role of 37LRP in R11 peptide uptake was tested. In several prostate cell lines examined, we only observed 37LRP but not

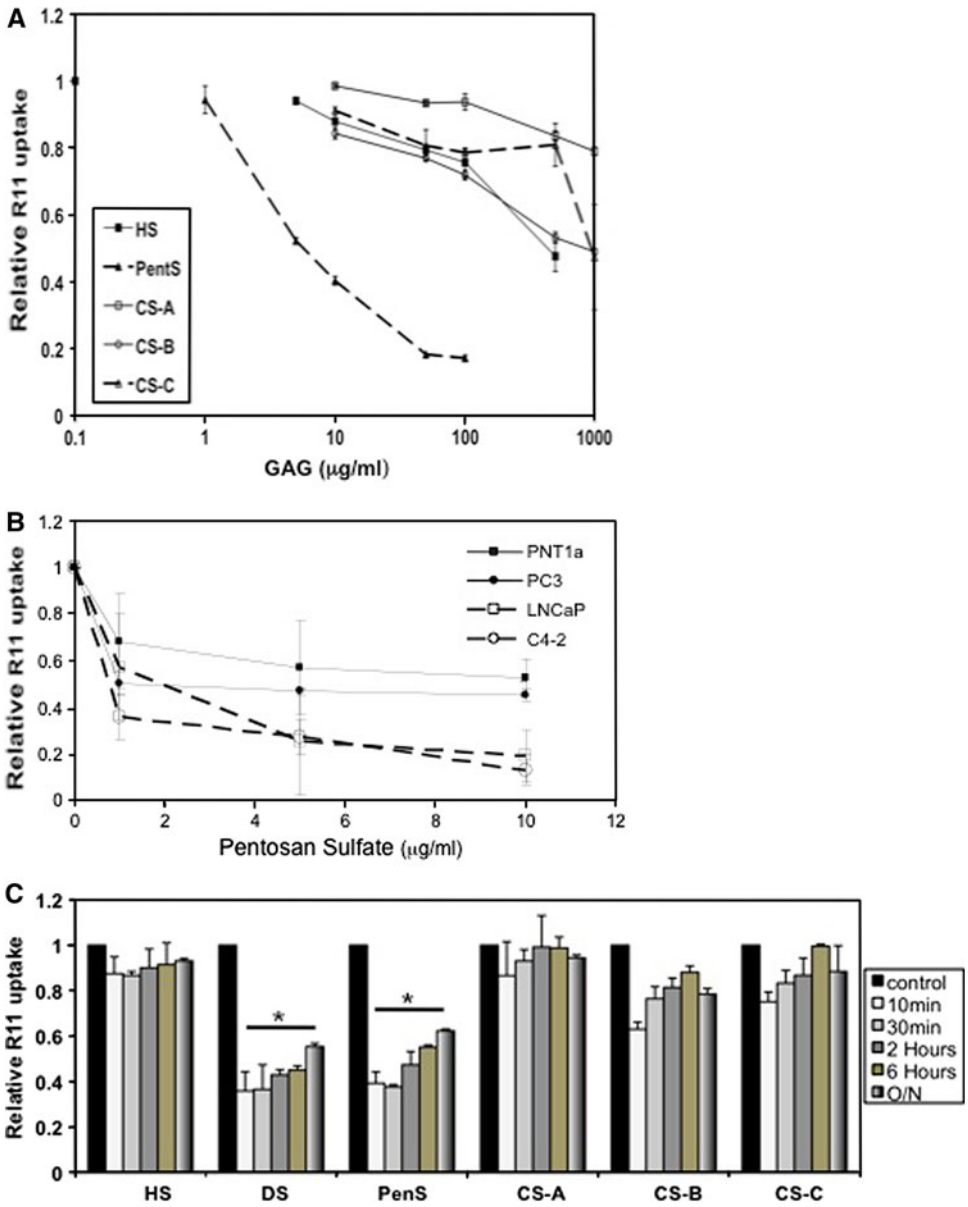

Fig. 3 The effect of soluble GAGs on R11 uptake in prostate cell lines. **a** Relative uptakes of R11 in PC3 cells was determined by co-incubating soluble glycosaminoglycans (GAGs) with 5 μmol/L of R11 for 30 min. **b** Effect of pentosan sulfate (PentS) on R11 uptake in various prostate cell lines. **c** Effect of GAGs on R11 uptake in various prostate cell lines by pre-incubation at different times. Different concentrations of GAGs were co-incubated with 5 μmol/L of R11 for 30 min. Relative R11 uptake from each sample was determined by FITC intensity divided by its protein concentration and then normalized with control. *Columns* mean in triplicate; *bars* SD. *Significant difference between control and each treatment ($P < 0.05$)

67LR as the major band from Western analyses, which may be due to two possibilities: 67LR is dissociated into 37LRP in the reducing condition during gel electrophoresis; 67LR expresses very low amount in prostate cancer cells. Thus, we examined whether R11 is able to associate with 37LRP in prostate cells. As shown in Fig. 4a, after incubating cells with FITC-R11 and then immunoprecipitating 37LRP using a specific antibody, it appeared that the binding of FITC-R11 to 37LRP exhibited a dose-dependent manner and this interaction was diminished by increasing the amount of competitive R11. In addition, the binding affinity of each oligo-arginine to 37LRP (Fig. 4b) was parallel with its cellular uptake (Fig. 1a). These data suggest that 37LRP is one of the primary "receptors" for R11 uptake by prostate cells.

Furthermore, 37LRP level in prostate cells was further manipulated using gene-specific siRNA. All four prostate cells were transiently transfected with 37LRP-specific siRNA and its control siRNA; mock transfection was used as a control. The efficiency of gene knockdown in each cell was determined using Western blot analysis of 37LRP expression levels after normalizing with a housekeeping protein (i.e., HSC70) and then compared with that of mock transfection. As shown in Fig. 5, a wide range of reduced 37LRP protein levels (20–80%) were present among these cell lines. R11 uptake was also determined from the same sample and was found to be 35–60% that of the mock transfection. The data suggest that 37LRP is one of the receptor(s) or a binding protein responsible for the uptake of R11 peptide by prostate cells.

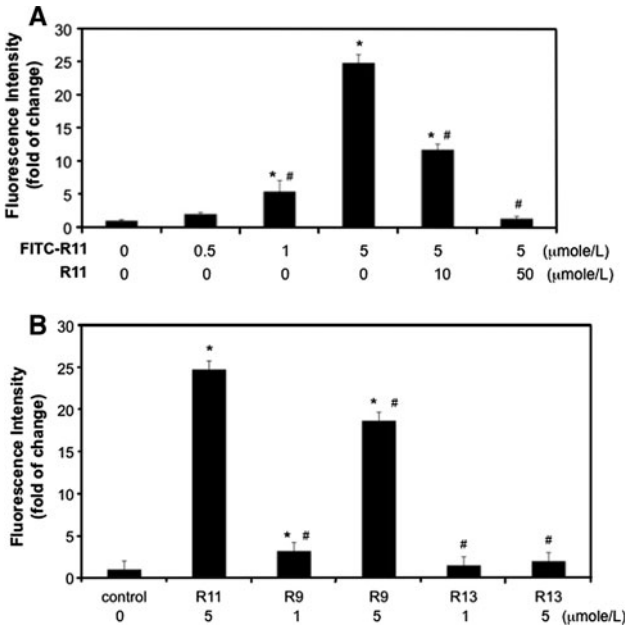

Fig. 4 The binding of R11 to 37LRP. **a** FITC-R11 or fluorescein was incubated with C4-2 cell line for 30 min incubation in the presence of competitive R11. **b** Various FITC-CPPs or fluorescein was incubated with C4-2 cell line for 30 min of incubation. After incubation, an equal amount of cell lysate (30 μg) was prepared from each treatment and subjected to immunoprecipitation with 37LRP antibody, and the immunocomplex was then determined by fluorometry. Relative fluorescence intensity from each sample was calculated by subtracting from control IgG and then normalizing with fluorescein control (=1). *Significant difference between control and each treatment ($P < 0.05$). #Significant difference between R11 (5 μmol/L) and each treatment ($P < 0.05$)

Discussion

R11 appears to be a highly efficient cell delivery vehicle compared with other CPPs in prostate cancers (Zhou et al. 2006) and an imaging probe for detecting metastatic prostate cells (Hao et al. 2010). It is also consistent with other findings that arginine-rich peptides exhibit higher delivery efficiency than other cationic homopolymers, such as histidine, ornithine and lysine (Mitchell et al. 2000). This higher efficiency has been attributed to the presence of the guanidine head group. In this study, we have found that R11 peptide has the highest uptake efficiency compared to R9 and R13 peptide in all prostate cells tested, including normal and cancer cells. We further analyzed the mechanism of R11 uptake in prostate cells.

While the mechanism(s) of uptake of CPPs is not fully understood; two possible mechanisms have been proposed. The non-endocytosis mechanism is proposed based on the observation that the uptake of CPP is in energy- or temperature-independent manner (Futaki et al. 2007). Also, using liposome model system, CPP has been shown to cross the lipid bilayer, which supports the non-endocytosis mechanism (Thoren et al. 2000). On the other hand, endocytosis mechanism is based on the observations that the uptake of CPP exhibits a punctuated pattern inside the cell resembling cell internalization, and the uptake of CPP is sensitive to endocytosis inhibitors (Futaki et al. 2007). However, recent studies have indicated that both mechanisms are not mutually exclusive (Fretz et al. 2007; Futaki et al. 2007). The contribution of either mechanism may depend on several factors such as cell types, individual CPP, culture condition and the physical/chemical property of payload. The subcellular distribution of R11 peptide with cytosolic or punctuated pattern (Fig. 1b) leads us to suspect that both mechanisms may also co-exist in prostate cells. Nevertheless, based on the inhibitory effect of EIPA on R11 uptake in prostate cells (Fig. 2), endocytosis mechanism should play a major role in this event.

Apparently, GAGs can affect the uptake of CPP. In the presence of soluble GAGs, the uptake of TAT protein is significantly inhibited (Tyagi et al. 2001; Nakase et al. 2007), suggesting that this inhibition is due to the competition of CPP binding between soluble and cell surface GAGs. Also, the membrane-associated GAGs, such as heparin sulfate proteoglycan (HPSG), are important for uptake of Tat peptide (Tyagi et al. 2001; Richard et al. 2005; Nakase et al. 2007). Pre-incubation of anionic polymers, such as dextran sulfate, can increase the uptake of oligo-lysine peptide (Tyagi et al. 2001). However, there is another indication that some CPPs, such as R8 peptide, are less dependent on HSPG, implying the existence of alternative cell surface binding site (Nakase et al. 2007). Our study has clearly demonstrated that the carbohydrate moiety of glycoproteins or carbohydrate chain elongation of proteoglycan failed to block R11 peptide uptake by prostate cells (Fig. 2), which further supports the idea that the cell surface binding site(s) for R11 peptide is very similar to GAGs interactive site(s).

The 37-kDa laminin receptor precursor (i.e., 37LRP) has been shown to bind to laminin and it can facilitate the interaction between laminin and integrins (Nelson et al. 2008). In addition, 37LRP protein has been suggested to play a role in the internalization of microorganism, as well as peptide and proteins. Some bacteria and viruses use the laminin receptor to enter into mammalian cells (Wang et al. 1992; Kim et al. 2005; Tio et al. 2005). The laminin receptor has been found to interact with prion protein identified from a yeast two-hybrid screening and that his interaction can be blocked by HS or PentS (Rieger et al. 1997). Moreover, laminin receptor has been shown to be a cell surface binding site for the internalization of the prion protein (Gauczynski et al. 2001). Interestingly, 37LRP is also involved in the binding of a CPP, PCK3145 that is derived from prostate secretory protein 94 (PSP94), as determined by a chemical analysis for the binding protein

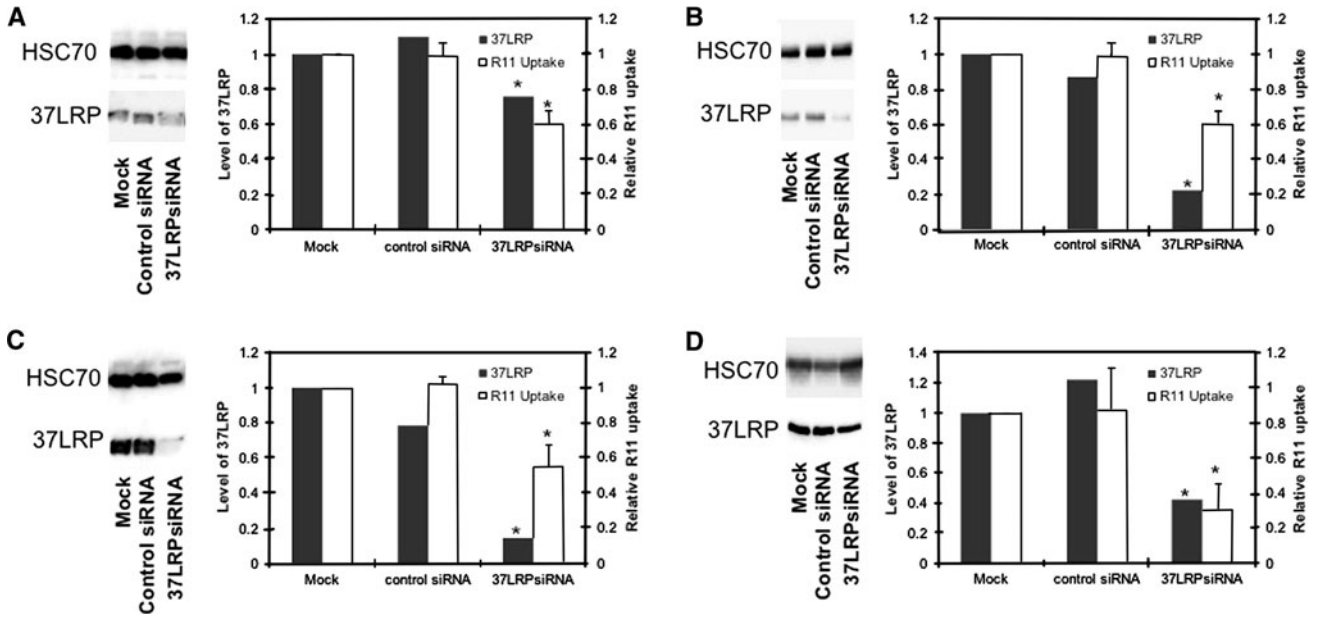

Fig. 5 The role of 37LRP in R11 uptake. PNT1A (**a**), PC3 (**b**), LNCaP (**c**) and C4-2 (**d**) cells were transfected with 37LRP siRNA or control siRNA for 4 days. R11 peptide (5 μmol/L) was added to each cell line for 30 min of incubation. The expression of 37LRP was determined using Western blot analysis and HSC70 was used as the internal control. The 37LRP protein level was quantified after normalizing with an internal control. The relative 37LRP protein level was calculated from each sample in triplicate after normalizing with the mock transfection. Relative R11 uptake from each sample in triplicate was determined by FITC intensity divided by its protein concentration and then normalized with mock transfection. *Bars* SD. *Significant difference between mock and each siRNA ($P < 0.05$)

of the PCK3145 peptide (Annabi et al. 2006). Our data indicate that R11 peptide uptake can be inhibited by soluble PentS (Fig. 3) that is known to inhibit the binding of 37LRP toward prion protein. Also, R11 can bind to 37LRP (Fig. 4), and R11 peptide uptake can be inhibited by 37LRP-specific siRNA in all the prostate cells tested (Fig. 5). Apparently, 37LRP is a primary binding molecule for R11 peptide uptake by prostate cells. Nevertheless, we cannot rule out the possibility of additional binding proteins responsible for this uptake because the observed inhibition of R11 uptake was not proportional to the reduced 37LRP expression levels in PC3 or LNCaP cell line (Fig. 5b, c).

Certainly, the uptake of CPP is mediated through complicated mechanisms, which are highly dependent on the cell type, the composition of CPP and perhaps its payload. We showed that the uptake of R11 peptide by prostate cells can be mediated via 37LRP by macropinocytosis. The entry of CPP peptide in vivo will be affected by the local extracellular micro-environments such as the composition of GAGs as well as the administration of the pharmaceutical reagents including carbohydrates such as PentS and HS. The results obtained from this study provide valuable information to determine whether R11 can be a potential delivery system for any given target cell or tissue. Also, understanding of uptake mechanism of R11 should help in the development of its application for any specific biomolecules.

Conclusion

Cell-permeable peptide is considered as an efficient delivery system to cross the cell membrane barrier. However, the underlying mechanisms are very complicated. Oligo-arginine peptide (i.e., R11) appears to have high affinity to prostate cancer cells compared with other peptides tested. We were able to develop R11 as an imaging probe to detect prostate cancer. In this study, we demonstrate that the uptake of R11 is mediated through macropinocytosis. However, inhibitors for carbohydrate synthesis of glycoprotein via either O-link or N-link, or inhibitor for carbohydrate chain elongation of glycosaminoglycan did not alter R11 uptake by prostate cells. In contrast, laminin receptor, a PentS binding partner, was able to influence R11 uptake in prostate cancer cells. Thus, we conclude that the laminin receptor is one of the initial binding sites responsible for R11 peptide uptake in prostate cells.

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